

Polyphenol oxidase/caffeic acid may reduce the allergenic properties of peanut allergens

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Abstract: Polyphenol oxidase (PPO) catalyzes the oxidation of tyrosine residues of proteins and, therefore, their cross-linking. Previously we demonstrated that cross-links produced by peroxidase (POD), which also catalyzes tyrosine oxidation, led to a reduction in the allergenic properties of peanut allergens.¹¹ We postulated in this study that PPO can also reduce the allergenic properties by cross-linking the allergens. Because caffeic acid, a phenolic compound, can cross-link proteins, its effect on peanut allergens was also examined. In the experiments, peanut extracts were treated with and without PPO, PPO/caffeic (pH 8, 37 °C for 1 h) and caffeic acid (pH 10.5, overnight), respectively. The samples were then analyzed for cross-links and IgE binding by SDS-PAGE, Western blots, and competitive inhibition ELISA. Results showed that, in all cases, cross-links and a decrease of the levels of two peanut major allergens, Ara h 1 and Ara h 2, were observed. Of the three treatments, PPO/caffeic was the most effective in reducing IgE binding or the allergenic properties of peanut allergens. The availability of tyrosine residues was also demonstrated in a POD-treated system, using a monoclonal antibody against dityrosine. We concluded that PPO/caffeic acid reduced the allergenic properties of Ara h 1 and Ara h 2 by cross-linking and decreasing the levels of allergens.

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Keywords: polyphenol oxidase; tyrosinase; caffeic acid; peanut allergens; Ara h 1; Ara h 2; cross-linking; IgE; dityrosine antibodies

INTRODUCTION

Polyphenol oxidase (PPO) or tyrosinase is a copper-containing enzyme widely distributed in fruits and vegetables.^{1,2} It catalyzes the oxidation of phenols to *o*-quinones (Fig 1a), which then polymerize with other phenolic compounds (eg caffeic acid) or amines to form browning products. Such browning reaction is often seen in fruits (eg apples) and vegetables (eg potato) when they are cut or damaged.^{3,4}

In proteins, PPO catalyzes the oxidation of tyrosine residue, which contains a phenol group, to an *o*-quinone derivative^{5,6} (Fig 1b). The resulting quinone on the protein (P₁) then reacts with the amino group, sulfhydryl group or the tryptophan residue of another protein (P₂) to form a protein-protein or P₁–P₂ cross-link (Fig 1b). Such a protein cross-link can also occur without tyrosine residue(s) if phenolic compounds such as caffeic acid or chlorogenic acid are present.^{5,6} As illustrated in Fig 1c, caffeic acid is oxidized by PPO into an *o*-quinone derivative. The quinone then reacts sequentially with the amino or sulfhydryl groups, or the tryptophan residues of proteins P₁ and P₂, thus generating a caffeic-containing

P₁–P₂ cross-link. In addition, cross-linking of proteins can occur without PPO if the reaction with caffeic acid takes place at pH 10 or above.^{7,8} Under this alkaline condition, caffeic acid is easily oxidized into an *o*-quinone derivative, and ultimately leads to the cross-linking of proteins P₁ and P₂ (Fig 1c).

It has been shown that treatment with PPO and/or caffeic acid can improve the functional properties of soy proteins,⁷ increase the heat stability of milk proteins,⁹ and enhance the anti-microbial effect of lysozyme.¹⁰ However, little is known about the effect of PPO and/or caffeic acid on peanut allergens. Previously, we have shown that the allergenic properties of peanut allergens can be reduced by peroxidase (POD).¹¹ The reason POD can reduce the allergenic properties is because POD induces cross-linking of the two major peanut allergens, Ara h 1 and Ara h 2,¹² by catalyzing the oxidation of their tyrosine residues. Unlike PPO, POD oxidizes tyrosine residue(s) of a protein (P₁) to a tyrosyl radical. This tyrosyl radical then couples with the tyrosine of another protein (P₂) to form a dityrosine-containing P₁–P₂ cross-link.^{11,13,14} As POD mainly targets the

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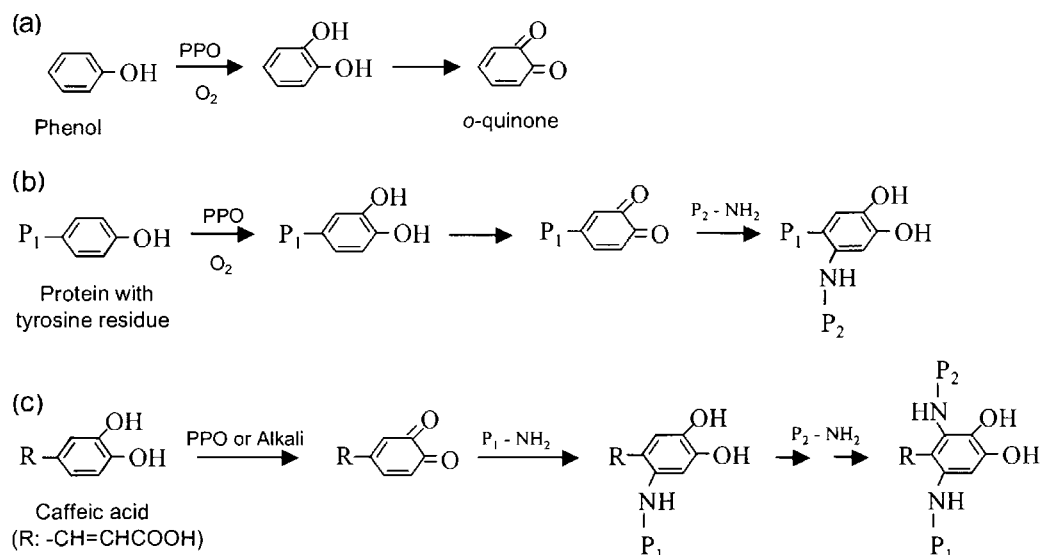


Figure 1. Reactions catalyzed by polyphenol oxidase (PPO) and/or caffeic acids. (a) phenol oxidation by PPO; (b) cross-linking of proteins P_1 and P_2 by PPO through tyrosine residues; (c) cross-linking of P_1 and P_2 by PPO in the presence of caffeic acid or by caffeic acid only at pH 10.5.

two major peanut allergens because of their tyrosine contents, it is reasonable to postulate that PPO may behave in the same way (ie cross-link specifically the allergens) and lead to the reduction of allergenic properties of allergens. In addition, we postulated that phenolic compounds such as caffeic acid may help reduce the allergenic properties because, as indicated above, caffeic acid, like tyrosine, can be converted into a quinone derivative and lead to cross-linking of allergens. In continuation of our investigation into the effects of various factors (eg Maillard reaction adducts, POD, polyamines) on peanut allergens,^{11,15–18} we determined in this study whether PPO and/or caffeic acid could induce the cross-linking of peanut allergens (Ara h 1 and Ara h 2), and thus reduce their allergenic properties.

MATERIALS AND METHODS

Chemicals

Polyphenol oxidase (25 000 units), bovine serum albumin (BSA), 96-well microtiter plate (Corning), rabbit anti-human immunoglobulin E (IgE)-peroxidase, o-phenylenediamine, Tween 20, 4-chloro-1-naphthol, rabbit anti-mouse IgG-peroxidase and phosphate buffer saline (PBS) were purchased from Sigma Co. (St Louis, MO). 4–20% Tris-glycine pre-cast gels were purchased from Invitrogen (Carlsbad, CA). Immobilon-P membrane was obtained from Millipore Corp. (Bedford, MA). Monoclonal anti-dityrosine antibodies (IC-3) were obtained from the University of Hyogo (Himeji, Hyogo, Japan). Human sera from five patients with peanut allergy were obtained from the University of Arkansas, Children's Hospital (Little Rock, AR). Superblock blocking buffer, GelCode Blue Stain Reagent and bicinchoninic acid (BCA)-protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL). Raw and roasted high-oleic peanut

seeds (SunOleic) were obtained from the University of Florida, Gainesville, FL.

Treatment of peanut extracts with PPO, PPO/caffeic and caffeic acid

Extracts in 20 mmol L⁻¹ sodium phosphate buffer pH 8 were prepared as previously described from defatted meals of raw and roasted peanuts.¹¹ Concentration of proteins in the extracts was determined using the BCA kit assay. Treatment of extracts with PPO was performed by incubating extracts in a final protein concentration of 2 mg mL⁻¹ with PPO (1 μ L, 40 units) at 37 °C for 60 min (total volume = 60 μ L). Treatment with PPO/caffeic acid was performed in the same way except that caffeic acid in dimethylformamide (20 mmol L⁻¹) (3 μ L) was also added (total volume = 60 μ L). For caffeic acid treatment, extracts were diluted with 0.3 mol L⁻¹ NaOH and then incubated with caffeic acid (PPO absent) at 37 °C overnight. Controls were performed in the same way except that no PPO, PPO/caffeic or caffeic acid was added. After incubation, the treated and untreated samples (5 μ L, 2 mg mL⁻¹ each) were subjected to SDS-PAGE, in which case, Tris-glycine pre-cast gels (4–20%) and a Novex Gel electrophoresis apparatus were used.¹¹ After SDS-PAGE, gels were subjected to Western blots (see below) or stained with GelCode Blue Stain.

Detection of dityrosine-containing cross-links with monoclonal antibodies

Extracts were first treated with peroxidase (POD) according to method of Chung *et al.*¹¹ SDS-PAGE and Western blots (5 μ L, 2 mg mL⁻¹) were performed according to the method of Chung and Champagne.¹⁵ with some modifications. Briefly, POD-treated proteins from gels of SDS-PAGE were transferred to an Immobilon-P membrane and blocked with

a SuperBlock solution. Dityrosine-containing cross-links were then detected by incubating the membrane with monoclonal antibodies^{19–21} (IC-3) against dityrosine at a dilution of 1:1000 (30 min), an anti-mouse-IgG-peroxidase (1:6000) (30 min), and a substrate of 4-chloro-1-naphthol (0.5 mg mL^{-1}) in triethanolamine buffered saline containing 0.2 g L^{-1} hydrogen peroxide. All samples except the substrate were diluted in (SuperBlock): (PBS/Tween 20) (1:1).

Determination of IgE binding in Western blots and competitive inhibition ELISA

Blots were performed as described above except that a pooled serum containing IgE antibodies (1:20) from peanut-allergic individuals and a rabbit anti-human IgE-peroxidase (1:500) were used. Competitive inhibition enzyme-linked immunosorbent assay (ELISA) was carried out ($n = 3$) as previously described.¹¹ Briefly, peanut extracts ($50 \mu\text{L}$) ($0.1\text{--}100 \mu\text{g mL}^{-1}$) were mixed with a pooled serum ($50 \mu\text{L}$) and then incubated in an allergen-coated plate for 45 min. The plate was washed, and a rabbit anti-human IgE peroxidase conjugate ($100 \mu\text{L}$) was added. After 30 min incubation and wash, a substrate ($100 \mu\text{L}$) containing o-phenylenediamine (0.5 mg mL^{-1}), 0.3 g L^{-1} hydrogen peroxide in 0.1 mol L^{-1} citrate buffer, pH 5.5 was added. After 15 min incubation at 37°C , the reaction was stopped with 2 mol L^{-1} sulfuric acid ($50 \mu\text{L}$). Absorbance was read at 490 nm with a CERES 900C plate reader (Bio-Tek Instruments, Inc., Winooski, VT). All samples except the substrate were diluted in [Superblock]: [PBS/Tw 20] (1:1). In Fig. 6, the absorbance value of a sample containing IgE antibodies and the extract was represented by B, while B_0 represented the absorbance value of a control containing IgE only. Values are means \pm SD ($n = 3$). Statistical analyses were performed using Student's *t*-test at a $P < 0.05$ level of significance.

RESULTS AND DISCUSSION

Effect of PPO treatment

SDS-PAGE profiles of PPO-treated and untreated extracts from raw and roasted peanuts are shown in Fig 2. PPO treatment led to a decrease in the density of several protein bands. Bands with a molecular weight of approximately 63 and 18 kDa are most noticeable. These bands correspond to the peanut major allergens, Ara h 1 and Ara h 2. Their decrease in density (indicated by an arrow sign) was accompanied by the appearance of low and high molecular-weight smears assumed to be cross-links. These smears are thought to result from the oxidation and cross-linking of Ara h 1, Ara h 2 and/or other proteins by PPO. This assumption was made, based on the fact that a purified Ara h 1 or Ara h 2 can cross-link itself and form high molecular-weight smears when its tyrosine residues are oxidized by peroxidase (POD),¹¹ and that the level of the purified allergen decreased as a result. The involvement of tyrosine in the formation of smears

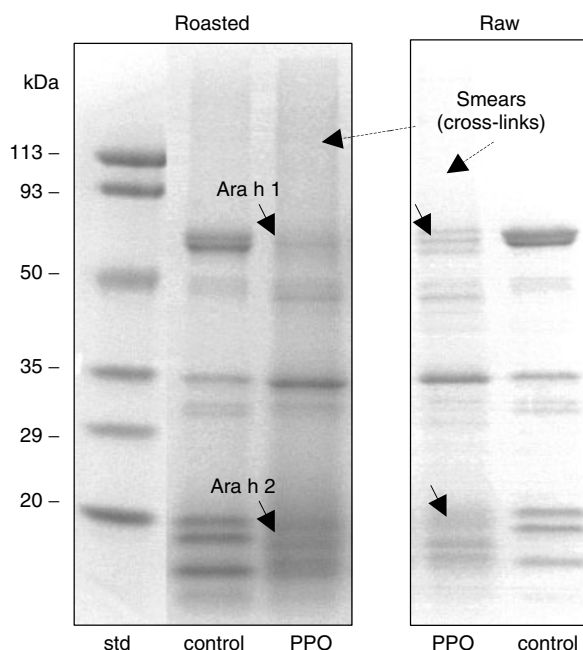


Figure 2. SDS-PAGE profiles of PPO-treated and untreated (control) extracts from roasted and raw peanuts. Smears or cross-links were formed as indicated. Change or disappearance of peanut major allergens, Ara h 1 and Ara h 2, is indicated by arrow signs.

and protein cross-links was further confirmed, using a monoclonal antibody against dityrosine (discussed below). Also, the association of the cross-links with the allergens was demonstrated, using immunoglobulin E (IgE) antibodies from peanut-allergic individuals (discussed below).

As shown in Fig 2 (SDS-PAGE), the cross-links formed by PPO, along with a decrease of Ara h 1 and Ara h 2 monomers, occurred in both treated raw and roasted peanut extracts. This suggests that tyrosine residues are accessible to PPO, regardless of raw and roasted conditions. However, in a previous study¹¹ using peroxidase (POD), which also oxidizes tyrosine but catalyzes cross-linking of peanut allergens in a different way, we saw cross-links only in roasted, POD-treated peanut extract. Raw peanuts did not form cross-links with POD but PPO, probably because of steric hindrance or repulsion which may prevent POD from reaching the tyrosine residues. Another possible reason for PPO being able to react with the allergens from raw peanuts is that phenolic compounds rather than tyrosine residues in the extracts may be involved. As illustrated in Fig 1c, phenolic compounds such as caffeic acids can be converted by PPO to a quinone derivative and lead to cross-linking of the allergens. Such compounds related to PPO are known to exist in peanuts,²² and their possible involvement in protein cross-linking may explain why PPO and not POD can form cross-links in raw peanut extract, even if tyrosine residues are not available.

Availability of tyrosine residues determined by monoclonal antibodies

In the case of roasted peanuts, tyrosine residues are probably available¹¹ and play a major role in

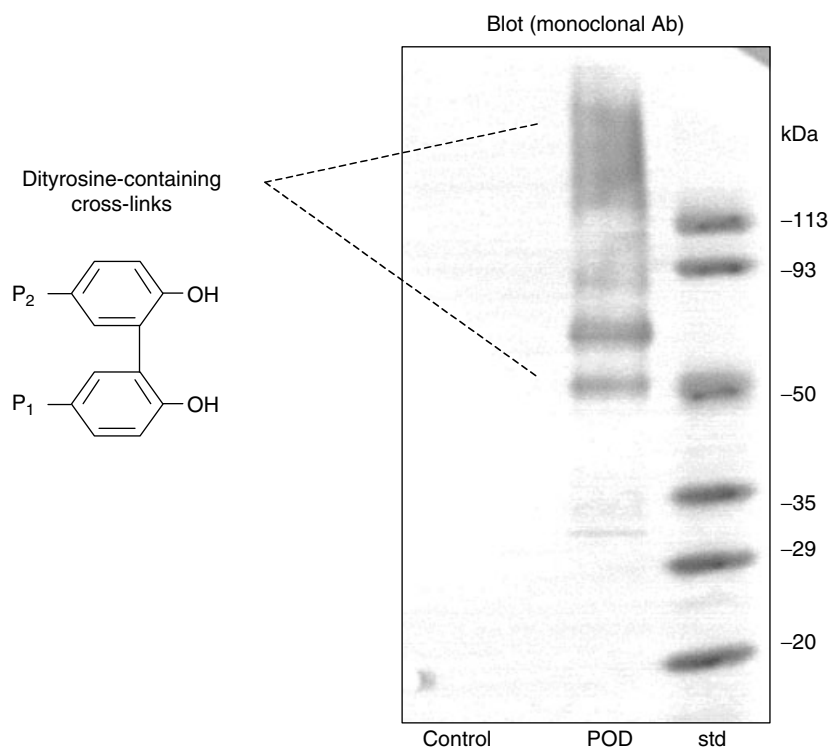


Figure 3. Detection of dityrosine-containing cross-links in Western blot. The purpose was to prove that tyrosine residues are available on the surface of roasted peanut allergens. Peroxidase (POD) was used in this case. When available, tyrosine residues of proteins (P₁ and P₂) can be oxidized by POD to form dityrosine-containing cross-links as shown. Cross-links were detected in blot, using a monoclonal antibody against dityrosine, a rabbit anti-mouse IgG-peroxidase, and a substrate of 4-chloro-1-naphthol.

the PPO-catalyzed cross-linking of peanut allergens. To determine the availability of tyrosine residues, we used peroxidase (POD) and a monoclonal antibody against dityrosine.^{19–21} Dityrosine is a dimer of tyrosine formed as a result of oxidation and cross-linking of two tyrosine residues. When tyrosine residues on the allergens are available and oxidized by POD, dityrosine-containing cross-links are formed¹¹ (see structure in Fig 3), and can be detected by the monoclonal antibody. In this experiment, we subjected roasted peanut extracts to POD/hydrogen peroxide, and then analyzed in Western blots (using the antibody) for the presence of dityrosine-containing bands thus formed. As shown in Fig 3, only the POD-treated sample, as compared with the untreated (control), had bands and smears that were detected by the antibodies. A band with a molecular weight (~63 kDa) similar to Ara h 1 was also detected. This band apparently was not Ara h 1 but a cross-link of other proteins and/or Ara h 2 because the control did not show Ara h 1. The finding demonstrates that tyrosine residues in roasted peanut allergens are available and responsible for the formation of cross-links. Based on this, we concluded that cross-links from the PPO treatment were products of tyrosine oxidation.

Because roasted peanuts may have less phenolic compounds,²² we used roasted peanuts for the rest of the study in which caffeic acid, a phenolic compound, was involved (discussed below).

Effect of PPO/caffeic acid treatment

As phenolic compounds may have a role in the cross-linking of peanut allergens, we postulated that addition of these compounds to the peanut extracts in the presence of PPO should result in an increase of cross-links along with an enhanced decrease of Ara h 1 and Ara h 2 allergens. To support our postulation, extracts were treated with and without caffeic acid in the presence of PPO. For comparison, the original extract (ie control with no PPO and caffeic acid added) was also shown. Results (SDS-PAGE) (Fig 4) show that while no change was seen in the control, both PPO/caffeic- and PPO-treated extracts produced smears (ie cross-links) and changes in band density (Ara h 1 and Ara h 2). In this case, bands were less dense (indicated by arrow signs), and smears were more intense in the PPO/caffeic-treated than in the PPO-treated. This means that PPO/caffeic was more effective than PPO in cross-linking the peanut allergens. In other words, caffeic acid helped enhance the cross-linking of Ara h 1 and Ara h 2 catalyzed by PPO. This finding seems to support the above assumption that phenolic compounds in raw peanut extracts may contribute, in the presence of PPO, to the formation of allergen cross-links.

Effect of treatment with caffeic acid only

In the absence of PPO, phenolic compounds are also known to be able to react with proteins. Such reaction is likely to take place under alkaline conditions^{7,8} and during processing of foods.^{9,23} Under alkaline

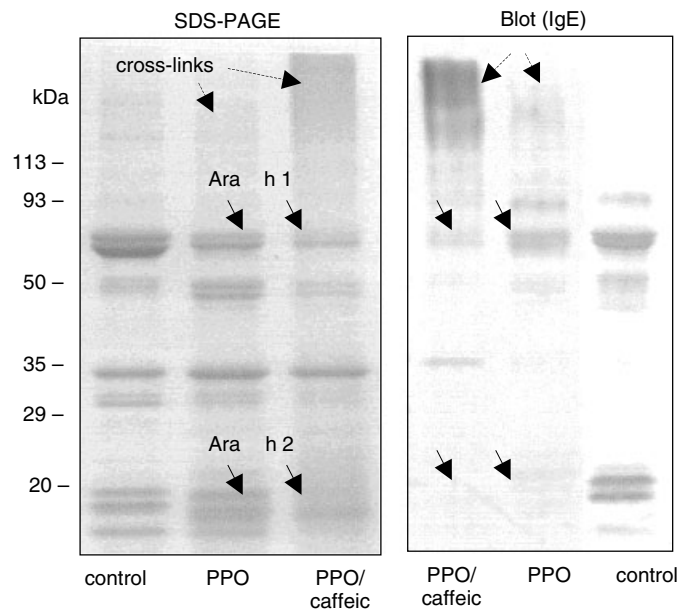


Figure 4. SDS-PAGE and Western blot (IgE) profiles of PPO- and PPO/caffeic-treated extracts from roasted peanuts. Extract without PPO or PPO/caffeic was used as a control. Change in band density, compared with the control, is indicated by an arrow sign. Bands in blot were detected, using a pooled serum (containing IgE antibodies) from peanut allergic individuals, a rabbit anti-human IgE-peroxidase and a substrate of 4-chloro-1-naphthol.

conditions, a phenolic compound such as caffeic acid is easily oxidized to a quinone derivative, and, therefore, leads to cross-linking of proteins (see Fig 1c). In order to determine whether caffeic acid can affect peanut allergens under alkaline condition, extracts were incubated with and without caffeic acid (no PPO in both cases) at pH 10.5 and 37 °C overnight. As shown in Fig 5 (SDS-PAGE), treatment with caffeic acid led to a significant decrease of Ara h 1 and Ara h 2 monomers. Also, low and high molecular-weight smears or cross-links were formed. This finding confirms the efficacy of caffeic acid in modifying peanut allergens under alkaline condition without PPO.

Effect of treatment on protein solubility

One concern was whether the above three treatments would cause a change in protein solubility. Such a change, if any, could lead to a change in the IgE binding measured below. We have shown previously that peanut allergens cross-linked by peroxidase (POD)¹¹ did not present a solubility problem. Here, using the same procedures previously described,¹¹ we examined the solubility of proteins in the PPO and/or caffeic-treated extracts. Briefly, we determined the clarity of the treated samples, the SDS-PAGE profiles of treated samples before and after centrifugation, and the SDS-PAGE profiles of treated samples in 4 mol L⁻¹ urea and without urea. Results showed that samples remained clear after PPO and/or caffeic treatment, and that samples processed by centrifuging and urea were not different in SDS-PAGE profiles from non-processed ones (data not shown). On this basis, we concluded that there was no protein solubility problem during treatments with PPO and/or caffeic acid.

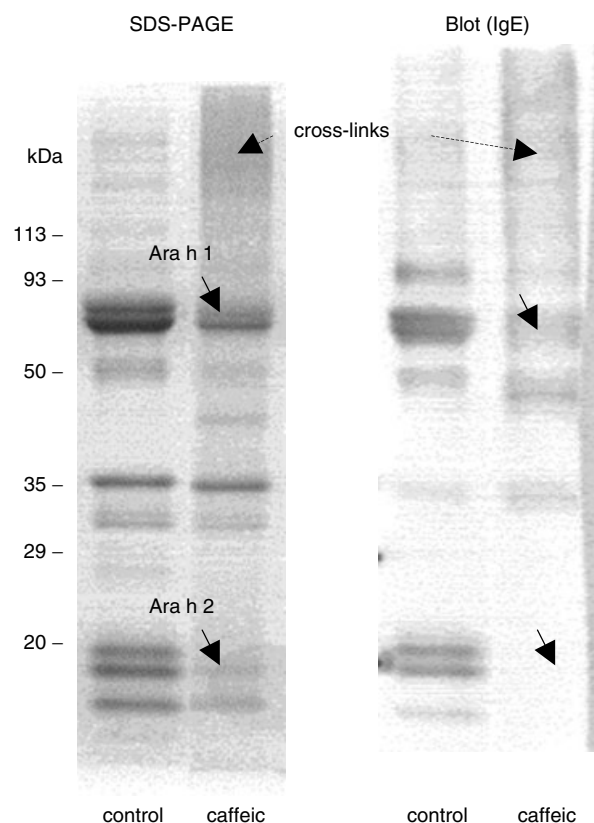


Figure 5. Profiles of caffeic-treated extracts from roasted peanuts in SDS-PAGE and Western blot (IgE). Detection is the same as described in Fig 4.

Effect on IgE binding or allergenic properties of treated-extracts

The final goal of this study was to determine whether extracts from various treatments (ie PPO, PPO/caffeic and caffeic acid only) display reduced IgE binding

or allergenic properties. First, Western blots were performed using a pooled serum (containing IgE antibodies) from peanut-allergic individuals. Results (Figs 4 and 5, blots) show that, in all cases (ie treated samples), bands of Ara h 1 and Ara h 2 were less distinct (particularly in PPO/cafeic-treated, Fig 4) than in the control. This suggests that IgE binding or allergenic properties could be reduced by PPO, PPO/cafeic or caffeic acid. However, a strong IgE binding to the cross-links (ie dark smears, particularly in PPO/cafeic-treated) was also observed in all treated extracts (Figs 4 and 5, blots). This indicates that there is a possibility that the cross-links may have the same degree of IgE-binding as the allergens and, if so, IgE binding, overall, was not reduced, because gain in IgE binding from the cross-links may compensate for the decrease in IgE binding from Ara h 1 and Ara h 2. However, it is also possible that, despite their binding to IgE, the cross-links may not be as allergenic as the original allergens, due to the IgE binding sites being altered by the treatments. In summary, the blot data could not tell whether there was a reduction, overall, in IgE binding in the treated extracts.

To determine whether there is indeed an overall reduction in IgE binding in the treated extracts, competitive inhibition ELISAs were performed using the same pooled serum. Figure 6 shows the inhibition of IgE binding by the control and individual treated samples. Table 1 shows the IC_{50} values of the control and treated samples, measured from the IgE inhibition curves from Fig 6. IC_{50} is the concentration of proteins in the treated extract or control required to inhibit IgE binding by 50%. So, the higher the IC_{50} , the less IgE inhibition or the less allergenic the proteins are. As shown in Fig 6a, PPO/cafeic-treated sample had a less pronounced IgE inhibition than the control, and, therefore, a higher IC_{50} value of $33 \mu\text{g mL}^{-1}$ than the control ($5 \mu\text{g mL}^{-1}$) (Table 1). This indicates that IgE binding or the allergenic properties of the PPO/cafeic-treated extract was significantly reduced. A similar but slight inhibition of IgE binding was also observed with the extract treated with PPO only (Fig 6a), which has an IC_{50} value of $14 \mu\text{g mL}^{-1}$ (Table 1). Comparison of the effects of PPO and PPO/cafeic indicates a more pronounced reduction of IgE binding in the latter. This suggests that PPO/cafeic is more effective than PPO in reducing IgE binding, and this agrees with the above SDS-PAGE data (Fig 4) that PPO/cafeic had a stronger effect on peanut allergens than PPO itself.

In addition, a less pronounced inhibition of IgE binding, compared with the control, by the extract treated with caffeic acid only at pH 10.5 was also noted (Fig 6b). The IC_{50} value for the caffeic-treated is $20 \mu\text{g mL}^{-1}$, compared with $5 \mu\text{g mL}^{-1}$ for the control (Table 1). This suggests that caffeic acid itself or related compounds (under alkaline condition) can lower the IgE binding or allergenic properties of peanut allergens.

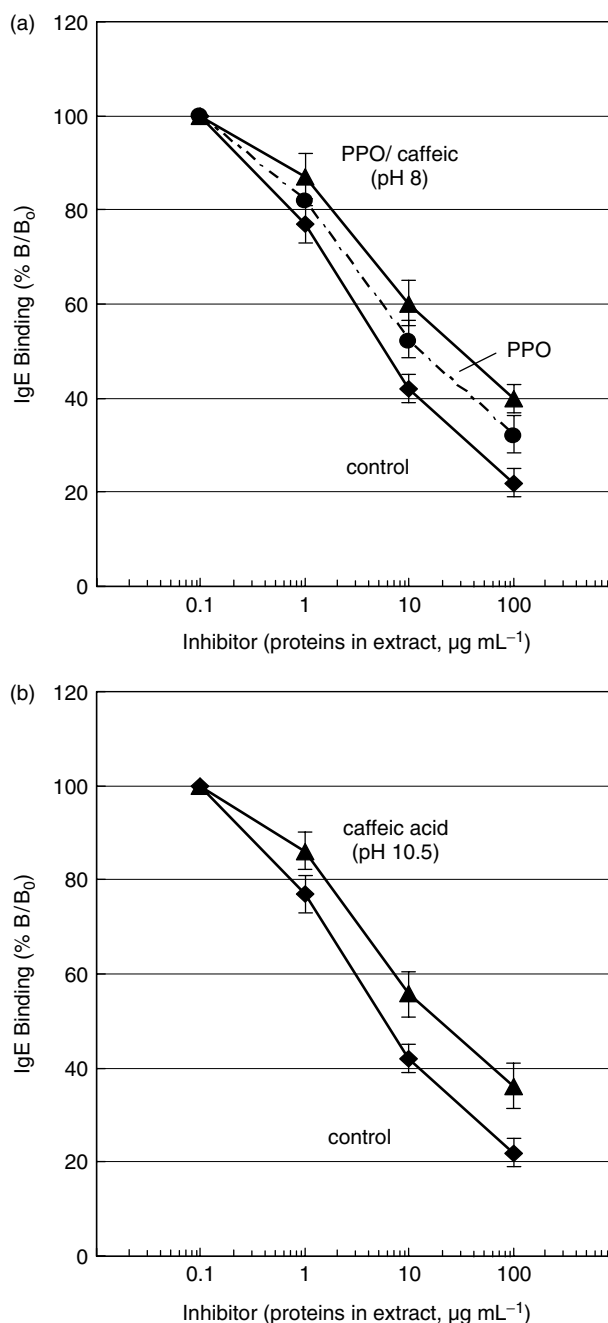


Figure 6. Inhibition of IgE antibodies in a competitive inhibition ELISA by (a) PPO- and PPO/cafeic-treated extracts, pH 8, and (b) caffeic-treated extract, pH 10.5. Extracts, diluted at the concentration indicated, were each mixed with a pooled serum from peanut allergic individuals, and then added to an allergen-coated microtiter plate. Detection of IgE antibodies was carried out using a rabbit anti-human IgE-peroxidase and a substrate solution of *o*-phenylenediamine and hydrogen peroxide. Values are means \pm SD ($n = 3$). Values of the treated samples at 1–100 $\mu\text{g mL}^{-1}$ are significantly different from those of the control ($P < 0.05$, $n = 3$).

On the basis of the IC_{50} values presented in Table 1, PPO/cafeic was the most effective of all treatments in reducing IgE binding of the peanut extracts.

CONCLUSIONS

We demonstrated that PPO, PPO/cafeic acid, and caffeic acid itself (under alkaline conditions) catalyzed

Table 1. IC₅₀ of treated samples^a

Treatment	IC ₅₀ (µg ml ⁻¹)
Control	5
PPO/caffeic	33
Caffeic, pH 10.5	20
PPO	14

^a IC₅₀ is the concentration of proteins required to inhibit IgE binding by 50% in Fig 6.

the cross-linking of peanut allergens and a decrease of the individual allergens. Tyrosine residues from the allergens and/or phenolic compounds in the peanut extracts were thought to play a major role in the formation of cross-links. The availability of tyrosine residues for such a role was confirmed, using monoclonal antibodies against dityrosine in a peroxidase-treated system. As a result of the PPO and/or caffeic treatment, IgE bindings or allergenic properties of peanut allergens were reduced. PPO/caffeic was the most effective of all treatments in reducing the allergenic properties of peanut allergens. While *in vivo* or molecular information about the cross-links is not given in the study, further research in this matter would help understand the allergenic properties of the cross-links.

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